Modulatory effects of the colonic milieu on neutrophil oxidative burst: A possible pathogenic mechanism of ulcerative colitis

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An important hallmark of ulcerative colitis (UC) is mucosal neutrophil (PMN) infiltration associated with mucosal damage. This suggests that colonic chemoattractants such as bacterial products (e.g., N-formyl-methionyl-leucyl-phenylalanine (fMLP), lipopolysaccharide (LPS)) reach systemic circulation and attract PMNs to the colon. PMNs are then activated in the colonic mucosa and release their toxic oxidative metabolites. However, bacterial products are also present in the systemic circulation of healthy subjects. Thus we hypothesized that PMNs develop tolerance to colonic factors in the normal state and that this tolerance is absent in UC. We evaluated the PMN respiratory burst in response to stimulation with fMLP, LPS, or phorbol 12-myristate 13-acetate (PMA) by measuring the production of reactive oxygen species (ROS) with both luminol-enhanced chemiluminescence and a cytochrome C reduction assay. PMNs were obtained from control subjects, inactive UC patients, patients with UC who had undergone colectomies, and non-UC patients with colectomies. All three stimuli induced a significant rise in ROS. PMNs from non-UC colectomy subjects produced significantly higher ROS than PMNs from control subjects with intact colons in response to both fMLP and LPS. In contrast, PMNs from UC colectomy patients produced levels of ROS similar to those produced by PMNs from UC patients with intact colons in response to fMLP and LPS. Colectomy had no effect on PMA-induced ROS production in controls. The observed difference in fMLP-induced ROS production in control subjects with intact colons was not due to fMLP receptor down-regulation because a competition assay performed with the fMLP blocker BMLP showed a similar receptor apparent affinity in all four groups. We conclude the following: (1) the normal colonic milieu modulates the PMN respiratory burst, resulting in hyporesponsiveness of PMNs to "physiologic" but not "pharmacologic" stimulation. This effect is not due to receptor down-regulation. (2) UC colonic milieu does not appear to modulate PMN respiratory burst. This loss of PMN "tolerance" to colonic factors may have a pathogenic role in the sustained inflammation and tissue damage in UC. (J Lab Clin Med 1997;130:216-25)

Abbreviations: BMLP = boc-methionyl-leucyl-phenylalanine; DMSO = dimethyl sulfoxide; fMLP = N-formyl-methionyl-leucyl-phenylalanine; LPS = lipopolysaccharide; MPO = myeloperoxidase; O^{-}_{2} = superoxide anion; PBS = phosphate-buffered saline solution; PBSG = PBS containing glucose, Ca^{++}, and Mg^{++}; PMA = phorbol 12-myristate 13-acetate; PMN = neutrophil; ROS = reactive oxygen species; SOD = superoxide dismutase; TNF = tumor necrosis factor; UC = ulcerative colitis

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Neutrophil functions such as phagocytosis and production of ROS[1] play an important role in host defense. Activated PMNs are attracted to sites of inflammation, where they defend the host against invading organisms. However, this useful process can become pathologic and cause tissue damage in certain inflammatory processes, including inflammatory bowel disease.[2-4]

UC is an inflammatory bowel disease characterized by multiple acute attacks of colonic inflammation alternating with periods of relative quiescence.[5] The acute phase of UC is characterized by the presence of acute colonic inflammation and intense mucosal PMN infiltration and tissue injury.[5] The most important hallmark of UC is that PMN infiltration and tissue damage are limited to the colonic mucosa. This histologic abnormality strongly suggests the presence of one or more factors in the colon that result in the attraction of PMNs to the colonic mucosa. This is not surprising, because the normal colonic lumen contains bacteria and bacterial products such as fMLP and LPS that are capable of attracting and activating PMNs.[6,7] In fact, one may argue that the lack of colonic inflammation in normal subjects is more surprising, because it has been demonstrated that small amounts of bacterial peptides such as fMLP do gain access into the circulation.[8,9] These observations suggest the possible development of a "relative tolerance" to colonic factors in the circulating PMNs in healthy subjects. We therefore hypothesized that factors in the colonic lumen of healthy subjects "down-regulate" the response of circulating PMNs to bacterial peptides, whereas this modulatory effect does not occur in patients with UC. To this end we evaluated the respiratory burst in response to bacterial peptides ("physiologic stimuli") in PMNs from subjects with intact colons and subjects who had undergone colectomies. We determined whether "normal" colonic factors blunt the ability of circulating PMNs to produce ROS by evaluating the function of circulatory PMNs from normal controls and subjects with subtotal colectomy and ileo-rectal anastomosis. We also investigated the role of colonic factors in modulating PMN production of ROS in UC by evaluating the respiratory burst in circulating PMNs from UC subjects with and without colectomies.

On observing that circulatory PMNs from control subjects with colectomies produced significantly higher levels of ROS in response to fMLP exposure, we studies a second group of subjects to further elucidate the mechanism of our finding. To determine whether our observed differences in fMLP-induced ROS production by PMNs between our patient groups were due to changes in fMLP receptor, we estimated the relative affinity of fMLP receptor on PMNs from the control and patient groups. Finally we measured LPS-stimulated PMN respiratory burst to determine whether differences in responsiveness could be observed with a second "physiologic" agonist that is typically present in the normal colonic lumen.

METHODS

Subjects

Control subjects. We originally included in the study 12 healthy control subjects with ages ranging from 24 to 58 years (mean = 35). Six were men and 6 were women. None were taking any medications. In the second part of our study, we evaluated 6 additional healthy volunteers (3 men, 3 women; mean age = 40).

Ulcerative colitis. We originally included in the study 17 patients with UC with ages ranging from 21 to 76 years (mean = 48). Eleven had active disease by standard clinical, endoscopic, and biopsy criteria as described previously.[10] Of these, 5 were men and 6 were women. Six had inactive disease characterized by the lack of rectal bleeding for at least 7 days and a normal colonoscopic appearance. Of these, all were women. All were completely off medications before the beginning of the study. Specifically, no subject had taken any immunosuppressive agents for 3 months, no subject had taken any steroids or antibiotics for 3 weeks, and no subject had taken any 5ASA or sulfasalazine for 1 week before the start of the study. In the second part of the study, we evaluated 5 additional patients with inactive UC (2 men, 3 women; mean age 48 years) who fulfilled the above mentioned criteria with regard to the diagnosis and medication.

UC with colectomy. We originally inlcuded in the study 6 patients with UC (2 men, 4 women), each having undergone total colectomy at least 1 year before the start of the study. All had undergone ileo-anal anastomosis. Ages ranged from 28 to 73 years (mean = 45). None were taking any medications. None had any clinical or endoscopic evidence of pouchitis. In the second part of the study, we evaluated an additional 6 patients (4 men, 2 women, mean age 53 years) with UC who had undergone total colectomy with ileo-anal anastomosis at least 1 year before the study.

Non-colitis colectomy. We initially included in the study six non-colitis subjects with sub-total colectomy. All had undergone subtotal colectomy with ileo-rectal anastomosis at least 1 year before the study and had rectal stumps between 13 cm and 20 cm. Three had subtotal colectomy for malignancy, and 1 each had subtotal colectomy for diverticular disease, angiodysplasia, and colonic bleeding of unknown pathogenesis, respectively. Their ages ranged from 58 to 81 years, with a mean of 69. All were men and none were taking any medications. For the second part of the study, we evaluated an additional 6 patients (mean age 64 years) with inclusion criteria identical to those in the

original 6 patients. All had their surgeries for colon cancer. Four were men and none were taking any medication.

Cell isolation. For the first part of the study, cell isolation was accomplished by a modification of the technique of Boyum[11] and Anton et al.[12] In brief, heparinized venous blood, drawn within 1 hour of the study, was diluted by 50% with saline solution, layered over Ficoll-Hypaque solution (Sigma Chemical Co., St. Louis, Mo.), and centrifuged at 400 *g* for 30 minutes at 4° C. The supernatant was discarded and the pellet, consisting of red blood cells and PMNs, was resuspended in sterile saline solution and mixed with a solution of 5% dextran-500. The suspension was allowed to sediment by gravity at 4° C for 30 minutes. The supernatant was again discarded and the cells were recovered by centrifugation at 150 *g* for 10 minutes. Lysis of the residual red blood cells was accomplished with ammonium chloride at a final concentration of 0.83%. The residual PMNs were washed twice with saline solution and resuspended in Hanks' buffer containing calcium and magnesium. PMN preparations were used immediately after isolation, and all studies were completed within 6 hours after the blood was drawn.

Cell isolation in the second part of the study was accomplished by a modification of the technique of Ferrente and Thong.[13] In brief, heparinized venous blood, drawn within 1 hour of the study, was layered over Polymorphprep solution (Gibco BRL) and centrifuged at 500 *g* for 30 minutes at 15° C. The PMN band was removed with a pipet, diluted by 50% with saline solution, and recentrifuged at 400 *g* for 20 minutes at 15° C. The supernatant was discarded and the pellet of PMNs was resuspended at a concentration of approximately 5×10^6 cells/ml in Hanks' buffer containing calcium and magnesium salts (pH 7.4). PMN preparations were used immediately after isolation, and all studies were completed within 6 hours after blood was drawn.

Both methods gave yields of approximately 10^7 PMNs per 10 ml of whole blood. The purity of the final preparations of PMNs obtained by the methods was >94%.

Chemicals. fMLP (Sigma) was dissolved in DMSO at a concentration of 100 ng/ml, aliquots were prepared, and stored at –70° C until used. On the day of testing, serial dilutions were freshly made in PBS such that final concentrations in the reaction mixtures were 1×10^{-5} mol/L, 10^{-7} mol/L, 10^{-8} mol/L, and 10^{-9} mol/L.

PMA (Sigma) was prepared at 10 μg/ml in DMSO, aliquots were prepared, and stored at –70° C. On the day of testing, serial dilutions were freshly prepared in PBS so that the final concentrations in the reaction mixtures were 10 ng/ml, 33 ng/ml, 100 ng/ml, and 333 ng/ml.

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) was prepared daily from a light-protected stock solution by dilution with PBS at a concentration of 1 μm. The dilution was stored in the dark and used immediately.

LPS (Sigma) was dissolved in phosphate buffer containing 1 mmol/L albumin, and aliquots were prepared and stored at –70° C until use. On the day of testing, the stock was diluted with PBS so that the final concentration in the microplate wells was 1×10^{-8} mol/L.

BMLP (Sigma) was dissolved in DMSO to a concentration of 1.0 μg/ml, and aliquots were prepared and stored at –70° C until use. On the day of testing, the stock was diluted with PBS so that the final concentrations in the microplate wells ranged from 1×10^{-5} to 1×10^{-11} mol/L.

SOD (Sigma) was dissolved in distilled/deionized water at a concentration of 3 mg SOD (3000 U = 1 mg protein) per milliliter. This solution was prepared weekly, and unused portions were stored for up to 1 week at 4° C.

Cytochrome C (from bovine heart) (Sigma) was dissolved in PBSG at a final concentration of 1.5 mmol/L. Unused portions of stable mixture was stored for 1 week at 4° C.

PBS was made by adding 1 part Hanks' balanced salt solution (–Ca^{++}, –Mg^{++}, HBSS 10× Gibco to 9 parts dH_2O).

PBSG was made by adding 99.89 mg $CaCl_2$, 101.6 mg $MgCl_2$, and 1.33 gm glucose to 1 L PBS.

Evaluation of PMN respiratory burst

Chemiluminescence studies. Luminol-enhanced chemiluminescence was used to estimate reactive oxygen metabolite production. The chemiluminescence spectrophotometer used in this study has been described by us previously.[10,14,15] Light was detected by an EMI 9813B photomultiplier in an EMI FACT 50 MK III cooler, cooled at –10° C and operated at 1375 volts. Single photon pulses were detected with an EMI APED amplifier discriminator (Thorn EMI, Ruislip, England), and these pulses were recorded with a frequency counter.

One milliliter of each PMN suspension, luminol, and stimulant were placed in a 12 mm × 75 mm test tube (total volume 3 ml), mixed gently by inversion, placed in the spectrophotometer, and preincubated for 1 minute. Light production was then measured every minute thereafter for 10 to 20 minutes until successive readings were similar. The maximum (peak) reading (in counts per minute) for each assay was used as an index of the respiratory burst. The peak or maximum rates were expressed as counts per minute per 10^6 PMNs.

Cytochrome C reduction assay. Superoxide anion production was measured by microplate kinetic assay as previously described by Mayo and Curnutte[16] and Chapman-Kirkland et al.[17] Before the start of the assay, the microplate reader, a 96-well microplate, the PBSG, and the cytochrome C solution were all equilibrated to 37° C. The following reagents were rapidly added to each well by a multichannel pipette: 170 μl PBSG, 12 μl cytochrome C solution (75 μmol/L), 5 μl SOD solution (60 μg/ml) or 5 μl water, and 50 μl cell suspension. Reagents were mixed by gently shaking the plate, which was then placed in an incubator (37° C) and allowed to equilibrate for 2 minutes. Superoxide anion (O^-_2) production of unstimulated cells was determined by recording the rate of change of the absorbance at 550 nm for 3 minutes and was used as the baseline. PMNs were

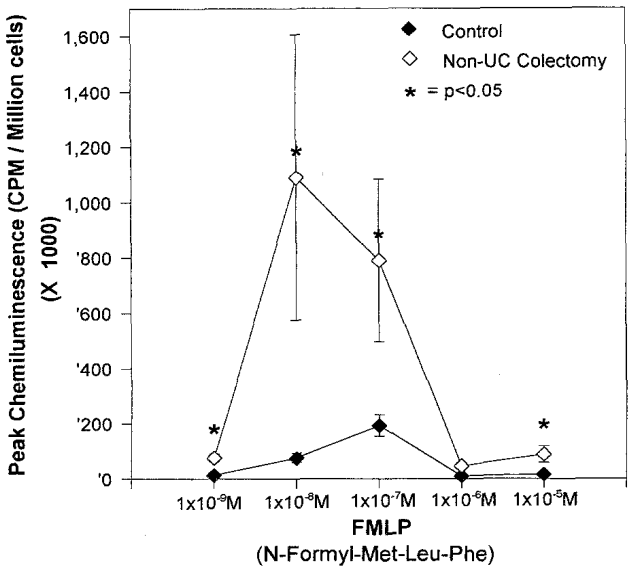

Fig. 1. fMLP-induced ROS production by PMNs from control subjects and subjects with non-ulcerative colitis after colectomy. Dose-response curves of peak PMN chemiluminescence after stimulation with five concentrations of the bacterial peptide fMLP are shown. There are statistically significant differences in peak luminol-enhanced chemiluminescence values between subjects with and without colons. PMNs from subjects without colons produced significantly higher levels of ROS than did PMNs from subjects with intact colons. *Brackets* represent SEM of the mean. $^*p < 0.05$.

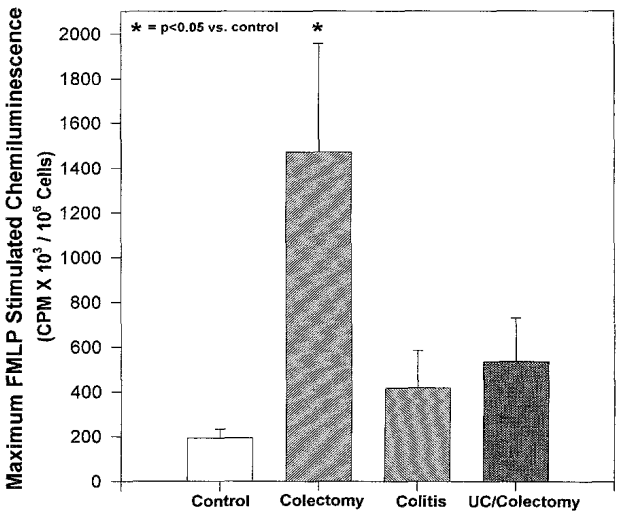

Fig. 2. Maximum fMLP-induced chemiluminescence production by PMNs from control subjects, patients with UC, non-UC patients after colectomy, and patients with UC after colectomy. PMNs from non-UC colectomy patients produced significantly higher levels of chemiluminescence than those from control subjects. Maximum fMLP-induced chemiluminescence values are expressed as the mean of the highest values in each patient regardless of fMLP concentration (typically 10^{-7} or 10^{-8} mol/L). *Brackets* represent SEM of the mean.

then activated by adding 6 μl fMLP (final fMLP, 10^{-7} mol/L) or 6 μl LPS (final LPS, 10^{-8} mol/L) to each well. Wells not receiving fMLP or LPS received 6 μl of PBSG so that the final well volumes were constant. When required, 6 μl of various dilutions of BMLP were added to produce final concentrations ranging from 10^{-5} mol/L to 10^{-11} mol/L. After cell activation, the absorbance change at 550 nm was monitored for 5 minutes, with intermittent agitation of the plate with a CERES II microplate reader (Biotech, Winooski, Vt.). The rates of O^-_2 production in each well were determined from the absorbance changes. The difference in the rates of cytochrome C reduction in the absence and presence of SOD is a measure of extracellular O^-_2 production, because one cytochrome C is reduced for every O^-_2 detected.[18]

In BMLP experiments, the rate of O^-_2 production in the absence of BMLP is considered 100%, and the percent change in the rate of O^-_2 production in the presence of various concentrations of BMLP was then calculated. A dose-response curve was generated for each experiment, and the BMLP concentration required for 50% inhibition was calculated by using a best-fit curve.

Statistical analysis. Group means were compared by using analysis of variance and Schefe's post-hoc test. Relationships between variables were evaluated by using Spearman's rank correlation. Data were considered significant if the probability value was less than 0.05.

RESULTS

PMN ROS production: Chemiluminescence studies

Non-UC patients with and without colons

FMLP STIMULATION. To evaluate the effect of endogenous, "physiologic" colonic factors, a bacterial peptide—fMLP—was used to stimulate the PMNs. fMLP at all doses significantly stimulated PMNs. The peak chemiluminescence occurred within 2 to 4 minutes, followed by a gradual decline in chemiluminescence for the remainder of the study period. There was no difference in the time course of fMLP-stimulated chemiluminescence between groups of patients with and without colectomy. Increasing concentrations of fMLP caused higher stimulation of PMNs to the maximum dose of 1×10^{-7} mol/L in control subjects and 1×10^{-8} in colectomy subjects (Fig. 1). Higher concentrations of fMLP had less-stimulatory effects in both groups. Peak chemiluminescence values in patients with colectomy were significantly higher, for doses of fMLP between 10^{-9} to 10 10^{-7} mol/L, than values in normal control subjects with colons (Fig. 1). Maximum chemiluminescence values (highest values in each patient regardless of fMLP concentrations) in colectomy subjects were significantly higher than those in control subjects with intact colons (Fig. 2). There was no statistically significant correlation between rectal

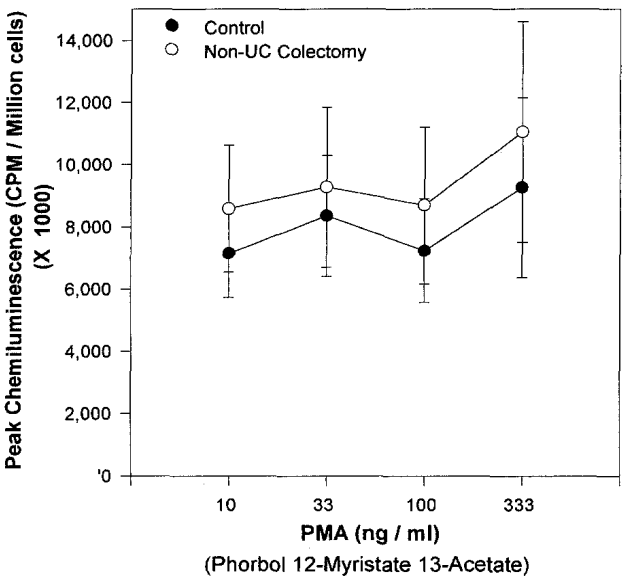

Fig. 3. PMA-induced ROS production by PMNs from control subjects and non-UC subjects after colectomy. Dose-response curves of peak neutrophil chemiluminescence after stimulation with four concentrations of a maximally activating PMN agonist PMA are shown. There are no statistically significant differences between two groups. *Brackets* represent SEM of the mean.

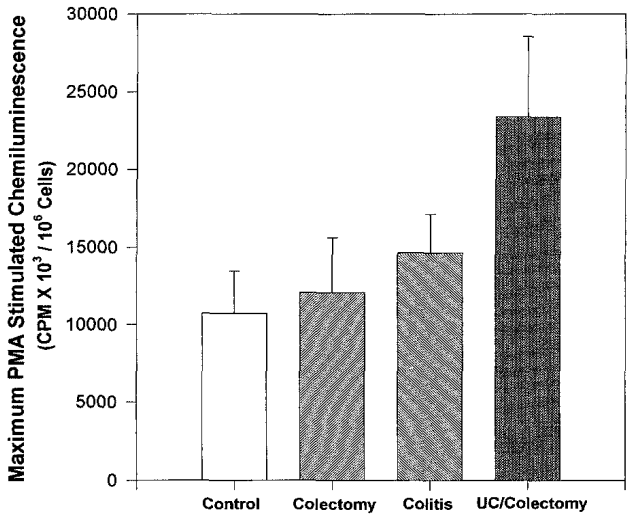

Fig. 4. Maximum PMA-induced chemiluminescence production by PMNs from control patients with UC, non-UC patients after colectomy, and patients with UC after colectomy. There was no statistically significant difference between groups. Maximum PMA-induced chemiluminescence values are expressed as the mean of the highest values in each patient regardless of PMA concentration.

stump length and peak chemiluminescence (r = 0.35).

PMA STIMULATION. To evaluate the effect of a nonphysiologic stimulus that is not present in the colonic lumen, PMA was used. PMA, at all doses, significantly stimulated PMNs. PMA was significantly more potent than fMLP as a stimulator. The peak chemiluminescence occurred after 12 to 18 minutes. There was no difference in the time course of PMA-stimulated chemiluminescence between the groups. In contrast to results with fMLP, no significant difference was seen in peak PMN chemiluminescence between subjects with and without colectomy (Fig. 3). There was no significant difference in the maximum chemiluminescence values (highest values in each patient regardless of PMA concentration) in colectomy subjects and subjects with intact colons (Fig. 4). Again, no correlation was seen between rectal stump length and peak chemiluminescence (r = 0.2).

Patients with UC with and without colons

FMLP STIMULATION. The patterns of fMLP stimulation in both UC groups were similar to those in control groups. fMLP-stimulated chemiluminescence in PMNs from patients with UC with intact colons was higher than that in PMNs from control subjects with intact colons, but the difference was not statistically significant. These data have been previously reported in detail.[19] Unlike in non-UC patients, colectomy in the patients with UC did not significantly affect the peak fMLP-induced chemiluminescence (Fig. 5). Maximum chemiluminescence usually occurred at an fMLP concentration of 1×10^{-7} mol/L both in patients with UC and post-colectomy patients with UC. There were no significant differences in chemiluminescence levels between the two groups at fMLP concentrations ranging from 1×10^{-6} mol/L to 1×10^{-9} mol/L. Furthermore, the maximum fMLP-induced chemiluminescence in non-UC colectomy patients was significantly higher than the maximum for UC post-colectomy patients (Fig. 2).

PMA STIMULATION. The PMA time course was similar to that in control subjects. There was no significant difference in PMA-stimulated chemiluminescence between control subjects and patients with UC with intact colons.[19] However, peak PMA-induced chemiluminescence in patients with UC after colectomy was significantly higher than that in PMNs from patients with UC with intact colons (Fig. 6). The maximum PMA-stimulated chemiluminescence in the UC colectomy group was 1.5-fold higher than that in the non-UC colectomy group, but this difference was not statistically significant (Fig. 4).

PMN superoxide anion production

Non-UC patients with and without colons

FMLP STIMULATION. fMLP at a concentration of 10^{-7} mol/L significantly stimulated O^-_2 production

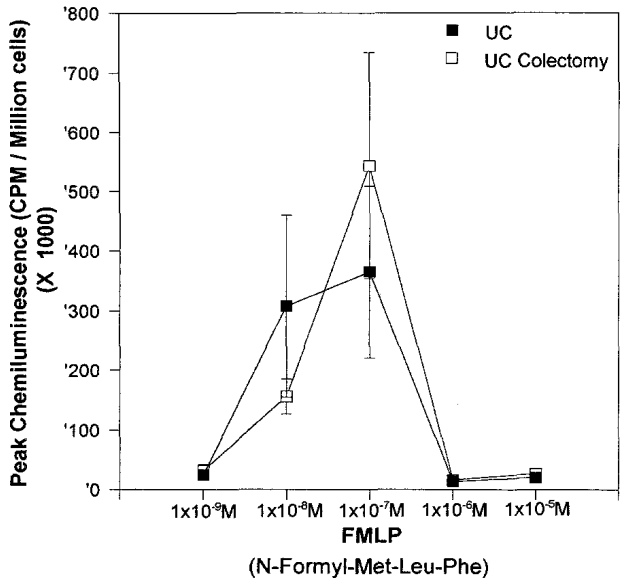

Fig. 5. fMLP-induced ROS production by PMNs from patients with UC and patients with UC after colectomy. Dose-response curves of peak PMN chemiluminescence after stimulation with five concentrations of fMLP are shown. There are no statistically significant differences between two groups. *Brackets* represent SEM of the mean.

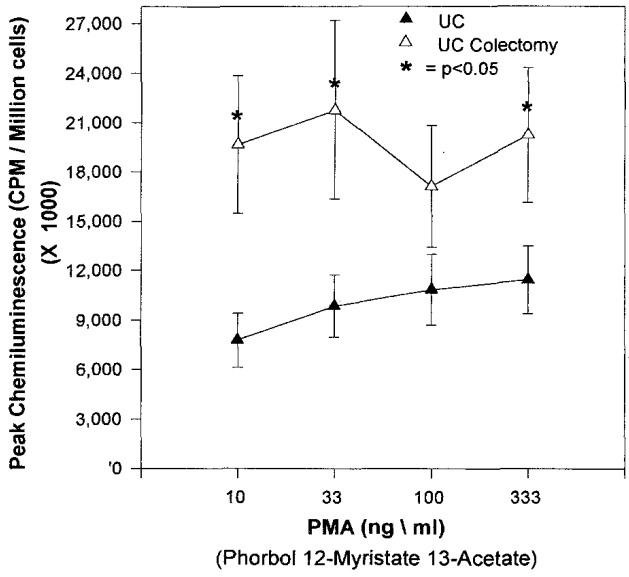

Fig. 6. PMA-induced ROS production by PMNs from patients with UC and patients with UC after colectomy. Dose-response curves of peak PMN chemiluminescence after stimulation after four concentrations of PMA are shown. PMNs from patients with UC after colectomy produced higher levels of ROS than did those from patients with UC with intact colons. *Brackets* represent SEM of the mean. $^*p < 0.05$.

by control PMNs to a level of 10-fold over baseline (0.4 ± 0.02 nmol/min/10^6 PMNs vs 4 ± 0.25 nmol/min/10^6 PMNs). Similar to the findings in ROS production (Fig. 2), PMNs from subjects with colectomies produced significantly ($p < 0.001$) higher levels of O^-_2 than did control PMNs (Fig. 7).

LPS STIMULATION. To determine whether the observed exaggerated PMN response to fMLP after colectomy was also present with a second "physiologic" stimulus, we evaluated the effect of colectomy on LPS-induced PMN respiratory burst. LPS significantly stimulated O^-_2 production by control PMNs to a level that was increased 35-fold over baseline (0.6 ± 0.02 nmol/min/10^6 PMNs vs 22 ± 2 nmol/min/10^6 PMNs). As in the case of fMLP-induced O^-_2 production, PMNs from subjects with colectomy produced twofold greater ($p < 0.001$) levels of O^-_2 than did PMNs from the control subjects (Fig. 8).

Patients with UC with and without colons

FMLP STIMULATION. fMLP at 10^{-7} mol/L significantly stimulated O^-_2 production by PMNs from UC subjects (7 ± 0.3 nmol/min/10^6 PMNs) and PMNs from UC subjects with colectomy (8 ± 0.2 nmol/min/10^6 PMNs). In contrast with results in the non-UC groups, colectomy had no effect on O^-_2 production by PMNs from the patients with UC (Fig. 7). These findings were thus similar to those obtained by the chemiluminescence technique (Fig. 2).

LPS STIMULATION. LPS also stimulated O^-_2 production by PMNs from UC subjects (Fig. 8). However, LPS stimulation was significantly greater in PMNs from patients with UC (36 ± 3.1 nmol/min/10^6 PMNs) than in PMNs from control subjects (22 ± 2 nmol/min/10^6 PMNs). As in the case of fMLP stimulation, colectomy had no significant effect on LPS-stimulated O^-_2 production by PMNs from patients with UC (Fig. 8).

FMLP RECEPTOR AFFINITY. We wanted to determine whether the observed differences in fMLP-induced O^-_2 production by PMNs from patients with colectomy was caused by changes in fMLP receptor affinity. To this end, an fMLP receptor blocker, BMLP, at four concentrations was used to quantitate relative receptor affinity. BMLP at a final concentration of 10^{-5} mol/L did not affect LPS-induced O^-_2 production by PMNs from control subjects (n = 3). BMLP concentration dependently inhibited fMLP-induced O^-_2 production by PMNs from all groups (Fig. 9). There were no statistically significant differences in BMLP concentrations required for 50% inhibition between control subjects (17 ± 4.9 nmol/L), UC (15 ± 6.8 nmol/L), non-UC colec-

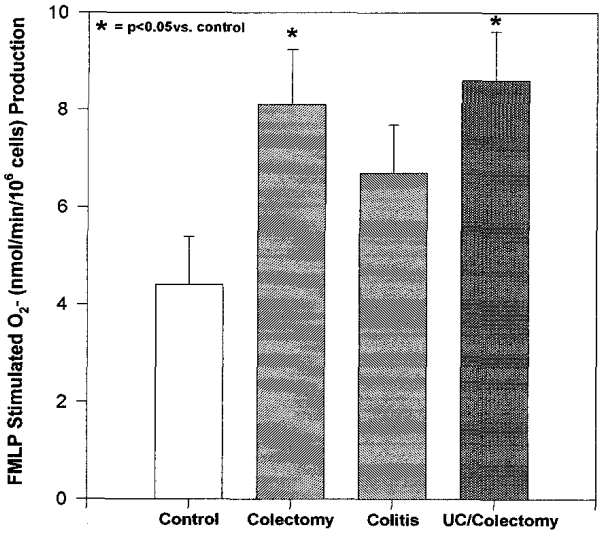

Fig. 7. fMLP-induced O^-_2 production by PMNs from control subjects, patients with UC, non-UC patients after colectomy, and patients with UC after colectomy. PMNs from non-UC patients after colectomy produced significantly ($p < 0.001$) higher levels of O^-_2 than did those from control subjects with intact colons. Colectomy had no significant effect on O^-_2 production by PMNs from patients with UC. *Bracket* represents SEM of the mean. fMLP concentration = 10^{-7} mol/L.

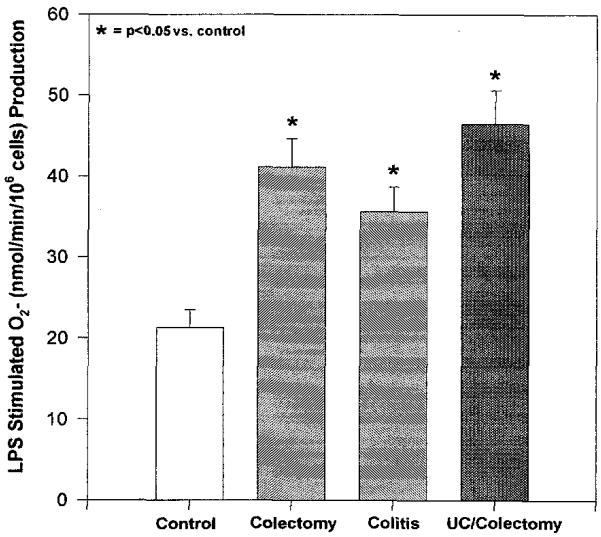

Fig. 8. LPS-induced O^-_2 production by PMNs from control subjects, patients with UC, non-UC subjects after colectomy, and patients with UC after colectomy. PMNs from subjects after colectomy produced significantly ($p < 0.001$) higher levels of O^-_2 than did those from control subjects with intact colons. Colectomy had no significant effect on O^-_2 production by PMNs from patients with UC. *Bracket* represents SEM of the mean. LPS concentration = 10^{-8} mol/L.

tomy (14 ± 5.6 nmol/L), and UC colectomy subjects (20 ± 5.2 nmol/L).

DISCUSSION

The pathogenesis of UC is not known.[5] It is possible that UC is not a single entity but a syndrome that is caused by several different pathogenetic factors. Even if UC is a consequence of various pathogenetic factors, it appears that there is one final common pathway to tissue damage. It is clear that a major component of this pathway is the PMN.[5,10,14]

Activated PMNs can cause tissue damage by producing toxic compounds such as the oxygen free radicals.[2] One of the most potent PMN activators is the bacterial polypeptide fMLP, which is in abundance in the normal colonic lumen.[20,21] It is also known that bacterial peptides can traverse the mucosal barrier and enter into the circulation,[8,9] especially when the barrier is disrupted to the degree that it is during the acute phase of UC. Thus, translocation of bacterial peptides might enhance the activation and recruitment of PMNs, creating a vicious cycle that results in the perpetuation of the inflammatory process and further mucosal damage. One possible mechanism that would prevent the initiation of such an inflammatory response in normal subjects is the development of "tolerance" of the host immune system to bacterial polypeptides.

The phenomenon of tolerance is now a well-accepted concept in the mucosal immune system of the gastrointestinal tract.[22] "Tolerant" mucosal lymphocytes in the gut have been demonstrated in normal subjects[23,24] and can be induced in circulating lymphocytes after incubation with normal mucosal extracts.[25,26] Several investigators have postulated that some gastrointestinal diseases including Crohn's disease[27] are the result of the absence of down-regulated lymphocytes with a lack of tolerance to luminal factors.

In contrast to cellular immunity and lymphocyte function where tolerance has been established, the notion of a "tolerant" PMN is not well demonstrated. However, several investigators have demonstrated that PMN function can be modulated by various factors to cause both hyperresponsiveness (priming) and hyporesponsiveness (tolerance) to subsequent exposures to agonists.[28-36] For example, TNF, interleukin-8, and bacterial peptides LPS/endotoxin and fMLP can prime PMNs.[28-34] Circulating primed PMNs have also been shown in a subgroup of anergic surgical patients.[37]

However, preincubation of PMNs with cytokines, as with TNF, does not induce priming under all conditions. For example, preincubation of PMNs in suspension with TNF resulted in a hyporesponsiveness as compared with TNF-induced stimulatory ef-

fects on adherent PMNs.[35] This "tolerance" was also induced by preincubation of PMNs with other factors such as LPS.[35] Furthermore, other compounds such as linolenic acid resulted in a hyporesponsiveness of PMNs to subsequent activation by agonists such as fMLP.[34] Additionally, tolerant PMNs have been noted in a subgroup of patients with myelodysplastic disease.[38] Circulating PMNs from these patients demonstrated suboptimal chemiluminescence signals when they were activated by fMLP, whereas their response to PMA was normal.

It is conceivable that abnormal PMN function or a lack of balance between "tolerant" and "primed" PMNs plays a mechanistic role in the creation of the sustained inflammatory process and tissue damage of UC. However, neither primed nor tolerant PMNs have ever been demonstrated in this disease. In the present study, our data strongly suggest the presence of such an imbalance. Our results suggest that PMNs from human subjects with intact colons are partially tolerant to the effects of the bacterial peptides fMLP and LPS. Circulating PMNs in non-UC patients with colectomies were significantly hyperactive and had an exaggerated response to fMLP and LPS as compared with responses in the control subjects with intact colons, suggesting that the presence of an intact colon may promote the development of tolerance. Such tolerance was not observed in PMNs from patients with UC, regardless of the presence or absence of the colon. Additionally, PMNs from patients with UC after colectomy produced significantly less chemiluminescence than PMNs from non-UC colectomy patients, suggesting that in patients with UC, the colon is a site for PMN priming rather than for the development of tolerance.

The observed hyperresponsiveness of PMNs from non-UC colectomy patients to fMLP and LPS might also be due to the priming of PMNs rather than to the development of tolerance. However, our previous in vitro observation[39] that preincubation of PMNs with normal rectal dialysates (which contain low levels of "physiologic" stimuli) blunt the stimulatory effects of fMLP suggests that the most important influence of the normal colon is to facilitate the development of tolerance. This notion is compatible with the findings of Schleiffenbaum et al.,[35] who demonstrated that low levels of LPS blunt the subsequent stimulatory effects of TNF. We also previously demonstrated that preincubation of PMNs with rectal dialysates from patients with active UC (which contain high levels of stimulatory substance) exaggerated the PMN response to the stimulatory effect of fMLP. Our present results suggest that PMNs from patients with UC are also primed to the

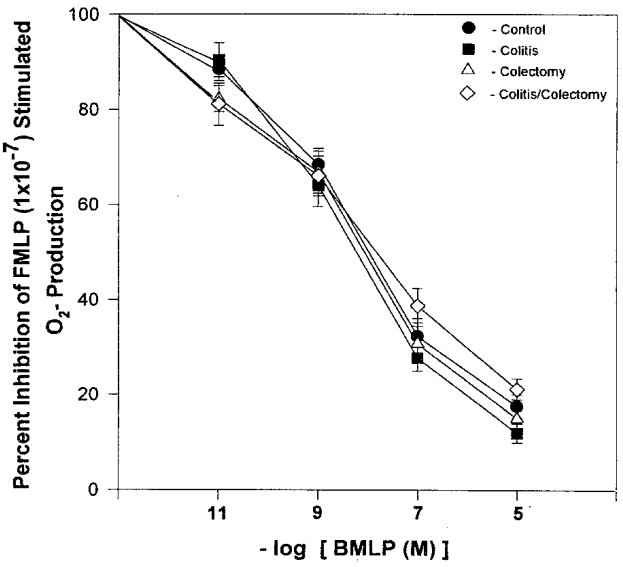

Fig. 9. BMLP inhibition of fMLP-induced O^-_2 production by PMNs from control subjects, patients with UC, non-UC colectomy patients, and UC-colectomy patients. All PMNs were preincubated with BMLP and then stimulated with fMLP (10^{-7} mol/L). Data (mean) represent percent inhibition of fMLP-induced O^-_2 production (in the absence of BMLP) by various concentrations of the fMLP blocker BMLP.

stimulatory effects of LPS. Our present data, which are supported by our previous in vitro findings, strongly support the hypothesis that PMN tolerance to stimulatory effects of luminal bacterial factors develops in normal individuals. On the other hand, such tolerance does not develop in the PMNs of patients with UC. Indeed, in these subjects the PMNs are primed to the stimulatory effects of luminal bacterial peptides.

The mechanism of the observed differences between PMNs from patients with UC and those from control subjects after colectomy is not known. It is possible that these differences are due to differences in the kinds and quantities of luminal factors present in UC and control subjects. Our previous in vitro rectal dialysate experiments support this possibility. It is also known that patients with inflammatory bowel disease have high levels of cytokines in their colonic tissue, even when their colons are not actively inflamed.[40] These high levels of stimulatory factors, in contrast to the low levels of "physiologic" stimuli, might induce priming rather than tolerance in PMNs in patients with UC. Indeed, we have recently demonstrated that the priming effects of rectal dialysates from patients with UC on PMNs are due to high levels of IL-8 that they contain.[41] Preincubation of rectal dialysates from patients with UC with anti-interleukin-8 antibody abolished the

priming effects of rectal dialysates on fMLP-induced PMN respiratory burst.[41]

Another possible cause for a PMN functional abnormality in UC is an intrinsic abnormality of the PMNs. However, this does not seem likely in view of prior studies that did not reveal any abnormalities in the circulatory PMNs in patients with UC.[19,42]

The cellular mechanism of PMN priming or tolerance (or both) in UC is not known. Recent studies indicate that changes in intracellular calcium concentration and the activation of tyrosine kinase enzymes may be involved in the signaling pathways that lead to PMN priming.[43,44] It also appears that receptor up-regulation may play a role in PMN priming in some[30,44] but not in all cases.[29,44] In the present study we demonstrated that fMLP receptor up-regulation is not responsible for hyperresponsiveness of PMNs from subjects with colectomy. Neither do changes in PMN function appear to be due to changes in reduced nicotinamide adenine dinucleotide phosphate oxidase complex, because the PMA-induced PMN respiratory burst was not significantly different in normal and hyperresponsive cells. Furthermore, a change in PMN MPO activity is not a likely explanation for these results, because both luminol-enhanced chemiluminescence (where MPO is a pivotal factor) and O^{-}_{2} production (where MPO is not a factor) were abnormal. Further studies are needed to evaluate the cellular mechanism of PMN functional changes after colectomy.

In conclusion, our data suggest that PMNs in control subjects appear to develop tolerance to stimuli that are normally present in the colonic lumen, such as the bacterial peptides fMLP and LPS. They therefore may be able to adapt to a daily exposure of small amounts of luminal stimuli without becoming activated. In contrast, this hyporesponsiveness disappears in UC, allowing the PMNs to become primed for a relatively exaggerated response to fMLP and other luminal bacterial peptides. Additional studies are, however, needed to further test this hypothesis in vivo and to explore the details of the interactions between colonic luminal factors and circulating PMNs in health and UC.

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